

REMARKS

Reconsideration is requested.

The specification has been revised to include a revised Sequence Listing containing the nucleotide and amino acid sequences of AB0007867.1 which can be found at the following internet site:

<http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucore&id=2662094>. The attached paper and computer readable sequences are the same. No new matter has been added.

The specification has been revised to correct an inadvertent typographical error.

Claims 76-114 are pending. Claims 76-105 and 112-114 have been withdrawn from consideration. Claims 1-75, 107, 108 and 110 have been canceled, without prejudice. Claims 106, 109 and 111 are under active consideration.

The Section 112, second paragraph, rejection and the Section 112, first paragraph "written description", rejection of claims 106, 109 and 111 are believed to be obviated by the above amendments. Entry of the present Amendment will obviate this rejections and therefore at least reduce these issues for appeal. Entry of the present Amendment and withdrawal of these Section 112, rejection are requested.

The Section 112, first paragraph "enablement", rejection of claims 106, 109 and 111 is traversed. Reconsideration and withdrawal of the rejection are requested in view of the following comments and the attached Wong et al ("Plexin-B1 mutations in prostate cancer" PNAS November 27, 2007, vol. 104, no. 48, 19040-19045).

The Examiner is understood to believe that the specification lacks experimental data which shows that the claimed mutations are involved in the etiology of cancer, so one of skill in the art could, according to the Examiner, not predictably use the claimed methods for identification of an anticancer drug without undue experimentation.

The Examiner is requested to see the attached Wong et al, which is a peer-reviewed publication co-authored by the present inventors which contains the mutation data which is set out in the instant specification. Wong et al also contains additional data which shows the functional effects of four separate plexinB1 mutations (A5359G; A5653G; T5714C and C5060T) in cultured cells.

All four plexinB1 mutants were shown to decrease the shrinkage or collapse of COS-7 cells relative to wild-type plexinB1 (Wong et al; figure 3c) and to significantly increase the adhesion of HEK293 cells relative to wild-type plexinB1 (Wong et al; figure 3d).

Furthermore, plexinB1 mutation was also shown to significantly increase the rate of migration of HEK293 cells relative to wild-type plexinB1 (Wong et al; figure 4a) and to increase the invasive capacity of HEK293 cells relative to wild-type plexinB1 (Wong et al; figure 4b). Expression of plexinB1 mutants in HEK293 cells was also shown to significantly increase the percentage of cell spreading and average cell size relative to expression of wild-type plexinB1 (Wong et al; figures 5a and 5b).

In addition, mutation of plexinB1 is also shown to inhibit RacGTP and R-Ras binding (Wong et al; figures 5c, 6a and 6b), which may contribute to the observed increase in cell adhesion and motility (Wong et al; page 19044 col 1 2nd para).

The functional data set out in Wong et al provides further confirmation that plexinB1 mutation is functionally important in the etiology of cancer, and in particular cancer progression. For example, Wong et al states at page 19044 col 1;

Together these results suggest that Plexin-B1 has a role in prostate cancer progression.

Wong et al further state the following at page 19044 col 2;

Plexin-B1 is likely to be a key player in cancer invasion and metastasis and is a potential target for anticancer therapy.

It is therefore evident that plexinB1 mutations are involved in the etiology of cancer. The claimed methods could therefore be predictably used by one of ordinary skill in the art for identifying a compound as a putative anti-cancer agent.

The claims are submitted to be supported by an enabling disclosure. Withdrawal of the Section 112, first paragraph, rejection is requested.

The claims are submitted to be in condition for allowance and entry of the present Amendment and a Notice of Allowance are requested. The Examiner is requested to contact the undersigned, preferably by telephone, in the event anything further is required in this regard.

WILLIAMSON ET AL
Appl. No. 10/536,804
Atty. Ref.: 620-373
Amendment After Final Rejection
December 24, 2008

Respectfully submitted,

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Plexin-B1 mutations in prostate cancer

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Edited by Stanley M. Gardler, University of Washington, Seattle, WA, and approved September 28, 2007 (received for review March 19, 2007)

Semaphorins are a large class of secreted or membrane-associated proteins that act as chemotactic cues for cell movement via their transmembrane receptors, plexins. We hypothesized that the function of the semaphorin signaling pathway in the control of cell migration could be harnessed by cancer cells during invasion and metastasis. We now report 13 somatic missense mutations in the cytoplasmic domain of the Plexin-B1 gene. Mutations were found in 89% (8 of 9) of prostate cancer bone metastases, in 41% (7 of 17) of lymph node metastases, and in 46% (41 of 89) of primary cancers. Forty percent of prostate cancers contained the same mutation. Overexpression of the Plexin-B1 protein was found in the majority of primary tumors. The mutations hinder Rac and R-Ras binding and R-RasGAP activity, resulting in an increase in cell motility, invasion, adhesion, and lamellipodia extension. These results identify a key role for Plexin-B1 and the semaphorin signaling pathway it mediates in prostate cancer.

adhesion | migration | R-Ras | Rac | semaphorin

Semaphorins are a large class of secreted or membrane-associated proteins that act as chemotactic cues for cell migration and axon guidance (1). Although best characterized in the nervous system, they are widely expressed in other tissues where they have a role in angiogenesis, organogenesis, and immune cell regulation (2, 3). Semaphorins inhibit integrin-mediated adhesion (4) and act as chemorepellants, although some also act as chemoattractants.

Plexins are single-pass transmembrane receptors for semaphorins (5, 6). Nine vertebrate plexins have been identified, grouped into four subfamilies, Plexin-A to Plexin-D, according to structure (5–7). Semaphorin 4D (Sema4D) is the ligand for Plexin-B1 and is expressed in both membrane-bound and secreted forms (6, 8). The cytoplasmic domain of plexins is conserved between plexin family members but has low homology to other receptor proteins (5) and lacks intrinsic tyrosine kinase activity. Plexins have a role in the regulation of the actin cytoskeleton, controlling the activity of the small GTPases, Rnd1, R-Ras, Rac, and Rho (9–12). Plexins also interact with and activate the receptor tyrosine kinases, ErbB2 (13) and c-Met (14). Plexins activate many characteristics of the invasive phenotype seen in cancer progression, inducing changes in extracellular matrix (ECM) adhesion, motility, scatter, and branching morphogenesis (15, 16). Prostate cancer is the second leading cause of cancer-related death in men in the U.S. and U.K.

We hypothesized that the function of the semaphorin-signaling pathway in control of cell migration could be harnessed by the cancer cell during invasion and metastasis. Our findings suggest that mutation or overexpression of the Plexin-B1 gene contributes to prostate cancer progression.

Results

Mutation of Plexin-B1 in Prostate Cancer Cell Lines. We hypothesized that mutations in the cytoplasmic domain of the semaphorin receptors, plexins, would be most likely to affect semaphorin signaling. We therefore focused our screen for mutations on stretches of the receptor genes encoding the cytoplasmic domain.

The cytoplasmic domain and part of the extracellular domain (exons 1–5) of plexins A1, A3, and B1 were sequenced using cDNA from the human prostate cancer cell lines PC3, DU145, and LNCaP. A single nucleotide change (A5359G) was found in Plexin-B1 in LNCaP, which potentially changes threonine 1697 to an alanine. The A5359G mutation alters a BstZ171 site, and estimation of the relative intensities of the mutant and WT restriction digest products (Fig. 1*a* and *b*), as well as the genomic DNA sequence, suggests that the mutation is present in two of the three copies of chromosome 3p in LNCaP cells. LNCaP was derived from a patient of Caucasian origin, and the sequence change was absent from the DNA of 60 control individuals of a similar genetic background. The rest of the Plexin-B1 cDNA was sequenced in PC3, DU145, and LNCaP, and no further mutations were found. Quantitative RT-PCR showed high levels of Plexin-B1 in LNCaP (Fig. 1*c*) relative to the other prostate cancer cell lines lacking a mutation.

Mutation of Plexin-B1 in Clinical Samples of Prostate Cancer. SSCP analysis was used to screen exons 22–29 of the cytoplasmic domain of the Plexin-B1 gene in DNA microdissected from tumor tissue. Aberrant bands were excised from the gel and sequenced (Fig. 2). Nine prostate cancer bone metastases were screened, and 17 further missense mutations were found in eight (89%) of the samples. The mutations were absent from uninvolved tissue of the same patient where available (seven cases) and are therefore somatic [Table 1 and supporting information (SI) Fig. 7]. Seventeen lymph node metastases contained nine missense mutations in 7 of 17 (41%) of the samples (Table 1 and SI Fig. 7). Eighty-nine primary prostate tumors treated by radical prostatectomy were screened, including DNA from 9 samples of fresh tissue, and mutations were found in 41 of 89 (46%) samples (Table 1 and SI Fig. 7), including 5 of 9 samples of fresh tissue (Fig. 2*a*).

DNA was also extracted from eight samples of adjacent morphologically normal fresh tissue from the same patients as the fresh primary tumor samples. Two of eight apparently normal tissue samples contained a mutation. In both cases, the same mutation was present in the tumor and the adjacent histopathologically defined normal tissue. These findings suggest that, although retaining a normal morphology, the tissue may contain early changes associated with the development of prostate cancer or that contaminating tumor cells are present, as observed in other studies (17).

Mutations were confirmed by direct sequencing [i.e., amplifica-

Author contributions: O.G.-W.W. and T.N. contributed equally to this work. O.G.-W.W., T.N., I.O., C.Z., V.B., J.N., G.B., P.M., J.C., and M.W. performed research; R.S.D.B., S.R.B., M.F., and H.K. contributed new reagents/analytic tools; S.E., M.N., A.F., and J.R.M. analyzed data; J.R.M. and M.W. designed research; M.W. and J.R.M. wrote the paper.

Conflict of interest statement: A patent has been filed by M.W. and J.R.M. for mutations.

This article is a PNAS Direct Submission.

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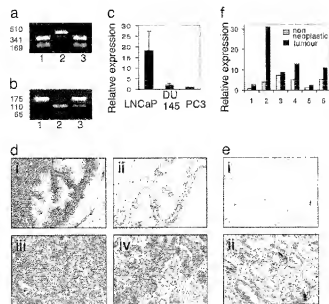


Fig. 1. Plexin-B1 expression in prostate cancer. (a) RT-PCR and Bst2171 restriction enzyme digestion of RNA from PC3 (lane 1), LNCaP (lane 2), and DU145 (lane 3). The A5653G mutation in LNCaP destroys a Bst2171 site. Band sizes in base pairs are shown. (b) PCR of genomic DNA and Bst2171 restriction enzyme digestion. Shown are undigested DNA (lane 1), PC3 DNA digested with Bst2171 (lane 2), and LNCaP DNA digested with Bst2171 (lane 3). The A5653G mutation in LNCaP destroys a Bst2171 site. (c) Quantitative RT-PCR of RNA from LNCaP, DU145, and PC3 cells. Results are the average of three independent RNA extractions normalized to PC3 expression. (d) Nonneoplastic (i) and primary cancer (ii and iii) tissue stained with H&E (i and ii) or antibody to Plexin-B1 (ii and iii). (e) Immunohistochemistry of prostate cancer tissue sections stained with anti-Plexin-B1 antibody to Sema4D. (Magnification, $\times 400$.) (f) Quantitative RT-PCR of RNA from paired samples of nonneoplastic tissue and primary cancer from the same patient (patients 1–6) normalized to nonneoplastic tissue from patient 1.

tion and sequencing of genomic DNA without isolation from a single-stranded conformation polymorphism (SSCP) gel or subcloning for the metastases (Fig. 2) and fresh primary tissue and by restriction enzyme digestion followed by sequencing for primary paraffin-embedded tissue (SI Fig. 8).

Some of the mutations were present in many tumors (Table 1). One mutation, A5653G (Thr1795Ala), was found in 9 of 26 (35%) metastases and 38 of 89 (43%) primary cancers. This mutation is predicted to result in the loss of a conserved predicted serine/threonine kinase phosphorylation site, C5060T (Pro159Leu) was found in five metastases and in four primary cancers, and mutation of the adjacent nucleotide (C5059T), which changes the same amino acid to serine, was found in three metastases and one primary cancer. T5714C (Leu1815Pro) was found in three metastases and in two primary tumors. Some of tumor samples analyzed contained more than one mutation, as has been observed previously for other genes in prostate cancer (18, 19). The sequence changes were absent from the DNA of at least 60 control individuals of a similar genetic background in all cases. Many of the mutations found in prostate cancer are at sites conserved during evolution, suggesting functional significance (SI Table 2).

Expression of Plexin-B1 in Prostate Cancer. To investigate the levels of expression of Plexin-B1 in prostate cancer, four replicate tissue microarrays (TMAs) of 85 samples of primary prostate cancer and 85 samples of nonneoplastic prostate tissue from the same radical prostatectomy specimens were stained for Plexin-B1 (Fig. 1*d*). A significantly higher proportion of cores stained positive for Plexin-B1 protein compared with the corresponding nonneoplastic

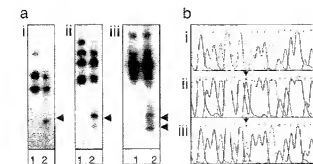


Fig. 2. Plexin-B1 mutations detected by SSCP analysis in prostate cancer. (a) SSCP analysis of nonmalignant DNA (lane 1) and DNA from a primary prostate cancer from fresh-frozen tissue (lane 2). The band marked with an arrow contains the C5060T sequence change. (b) SSCP analysis of nonmalignant DNA (lane 1) and DNA from a primary prostate cancer from fresh-frozen tissue (lane 2). The band marked with arrows contains the A5653G mutation. (c) SSCP analysis of nonmalignant DNA (lane 1) and DNA from a prostate cancer lymph node metastasis (lane 2). The bands marked with arrows contain the A5653G mutation. (d) Mutations in the Plexin-B1 gene. (i) Normal DNA sequence. (ii) Sequence of a SSCP band showing the A5653G mutation. (iii) Direct sequence of DNA from a metastasis in the same patient as in *bii*, showing the A5653G mutation.

tissues ($P < 0.001$, Mann-Whitney tumor vs. nonneoplastic, for both extent and intensity). Expression of Plexin-B1 was found in 77% (65 of 84) of prostate tumors and 6% (5 of 83) of nonneoplastic tissue from the same patients. All nonneoplastic cores were from patients diagnosed with prostate cancer. Consequently, the five cases of nonneoplastic tissue that did stain for Plexin-B1 may have had early changes associated with prostate cancer, although retaining normal morphology as has been described in other studies (20). Quantitative RT-PCR performed on paired samples of nonneoplastic and primary cancer showed increased expression of Plexin-B1 in the cancer samples relative to nonneoplastic tissue from the same patient (Fig. 1*f*). These results suggest that the increased protein staining observed in the primary cancers results from increased RNA levels and not from altered protein stability.

The same prostate cancer TMAs were also screened for protein expression of Sema4D, the ligand of Plexin-B1, by immunohistochemistry (Fig. 1*e*). Cell-surface expression of Sema4D was found in the basal epithelial cells of nonmalignant prostate tissue but was absent from the luminal cells. High levels of Sema4D cell surface staining were seen in prostate cancer cells, demonstrating a cancer-cell-associated overexpression of Sema4D ($P < 0.001$, Mann-Whitney, tumor vs. nonneoplastic, for both extent and intensity). Positive staining was found in 58% (49 of 84) of prostate cancers and 3.5% (3 of 84) of nonneoplastic tissues. A significant correlation was also seen between Plexin-B1 and Sema4D staining in the tumor samples ($P < 0.001$, Mann-Whitney). The same tumor cores also showed high levels of c-Met expression (data not shown).

Functional Significance of Mutations in Plexin-B1. To determine whether the mutations in Plexin-B1 found in prostate cancer metastases have an effect on cell function, cDNA constructs were made encoding WT Plexin-B1 and four mutant versions of the protein. The mutants investigated were the three most frequently found in prostate cancer metastasis, (C5060T, A5653G, and T5714C) and that found in LNCaP (A5359G).

Mutation of Plexin-B1 Decreases Cell Collapse. In the presence of Rnd, stimulation of Plexin-B1 with Sema4D results in a transient cell shrinkage or collapse of COS-7 cells (10). To determine the effect of Plexin-B1 mutations on this standard test of plexin function (21), COS-7 cells were transiently transfected with Rnd1 and WT or mutant Plexin-B1, and the cells were stimulated with

Table 1. Mutations in prostate cancer

Nucleotide	Amino acid	Bone metastases (total of 9)								LN metastases (total of 17)							1* (total of 89)	Total
		1	2	3	4	5	6	7	8	1	2	3	4	5	6	7		
C5059T	P1597S											x	x	x	x		1	4
C5060T	P1597L		x	x			x				x					x	4 [†]	9
G5074A*	G1602R																	1
T5401A	F1711I																1	1
C5468T*	T1733I							x										1
A5474G*	N1735S							x										1
A5596G	T1775A																1	1
A5593G*	T1795A	x	x	x	x	x			x	x				x	x		38 [†]	47
C5662T	T1798S								x									1
A5674G*	T1802A			x														1
T5714C*	L1815P		x						x								2 [†]	1
C5980T	R1904W									x								1
Total						8 of 9 (89%)							7 of 17 (41%)				41 of 89	73

*Mutation absent from noncancer tissue from the same patient in at least one case; the rest are not determined.

[†]DNA from frozen tissue.

[‡]Includes five samples of DNA from frozen tissue.

Sema4D. Coexpression of Rnd1 and WT Plexin-B1 and treatment with Sema4D for 5 min resulted in an increase in cell collapse as expected (Fig. 3c). In contrast, transient expression of all four Plexin-B1 mutants significantly reduced the percentage of cell collapse relative to expression of WT Plexin-B1 ($P \leq 0.05$, Fig. 3c).

Mutation of Plexin-B1 Increases Cell Adhesion. Expression of Plexin-B1 in fibroblasts inhibits integrin-dependent cell adhesion to the ECM protein fibronectin (22). This inhibition is due to activation of the intrinsic GTPase activity of R-Ras by Plexin-B1, resulting in R-Ras inactivation and a decrease in integrin-based cell

adhesion (9). To assess the effect of four of the mutations found in Plexin-B1 on cell adhesion, stable cell lines expressing WT or mutant Plexin-B1 were made in HEK293 cells by lentiviral transduction. The cell lines expressed similar levels of Plexin-B1 protein, and cell surface expression of WT and mutant Plexin-B1 was observed (Fig. 3a and b). Expression of WT Plexin-B1 reduced adhesion of HEK293 cells to fibronectin relative to a vector control 10 and 30 min after plating (Fig. 3d and SI Fig. 9a). Binding of Plexin-B1 by its ligand, Sema4D (6), results in clustering of the receptor (23), and expression of high levels of Plexin-B1 mimics ligand binding (14) (SI Fig. 9a). Probably for this reason, the addition of Sema4D had little additional effect on adhesion. Expression of all four mutants significantly increased adhesion relative to WT Plexin-B1 after 10 ($P \leq 0.005$) and 30 min ($P \leq 0.05$) (Fig. 3d and SI Fig. 9a). The addition of Sema4D reduced adhesion for all four mutants, but levels remained significantly above WT ($P < 0.05$). Integrin-independent adhesion to poly-L-lysine was negligible and was not affected by expression of any of the constructs (SI Fig. 9b).

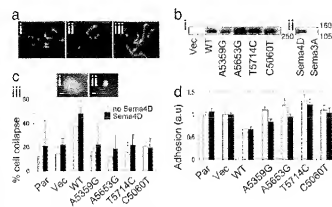


Fig. 3. Mutations in the Plexin-B1 gene inhibit cell collapse and increase cell adhesion. (a) Lentiviral-transduced HEK293 cells expressing WT Plexin-B1 (i), T5714C (ii), or C5060T mutants (iii), showing cell-surface expression. Cells were stained for Plexin-B1 (TRITC) and actin (FITC-phalloidin). (Scale bar, 10 μ m). (b) Western blot of lentiviral-transduced HEK293 cells infected with vector (Vec), WT, or mutant Plexin-B1, selected using blasticidin, and detected by anti-Plexin-B1. (i) Western blot of purified Sema4D-4C and Sema3A-4C. (ii) and (iii) Mutations in Plexin-B1 inhibit cell collapse. COS-7 cells transfected with Rnd-myc and mutant (A5593G) or WT Plexin-B1 (i) and treated with Sema4D. Cells were stained for myc (FITC) and Plexin-B1 (TRITC). (Scale bar, 10 μ m). (ii) Percentage of cell collapse of cells transfected with Rnd and Plexin-B1 (WT or mutant) and treated with Sema4D for 5 min. The size of cells transfected with both constructs was measured, and the percentage of cells $<500 \mu$ m² was calculated using Andor software. The results are the mean of three independent experiments, with 50 cells sized per experiment. $P \leq 0.05$ (all mutants vs. WT). Error bars indicate SEM. (d) Mutations in Plexin-B1 increase adhesion. Shown is adhesion of lentiviral-transduced HEK293 cells expressing WT or mutant Plexin-B1 to fibronectin for 10 min. No Sema4D, $P \leq 0.005$ (all mutants vs. WT); with Sema4D, $P \leq 0.01$ (C5060T, A5593G, and T5714C) and $P \leq 0.05$ (A5593G) vs. WT.

Mutation of Plexin-B1 Increases Cell Motility and Invasion. Plexin-B1 expression reduces cell motility in NIH 3T3 and COS7 cells (22, 24) and increases cell motility in cells expressing c-Met (14). To assess the effect of mutation of Plexin-B1 on cell motility, the rate of migration of HEK293 cells expressing WT or mutant Plexin-B1 was measured by time-lapse video microscopy. Expression of WT Plexin-B1 reduced cell motility relative to vector controls (Fig. 4a). Expression of all four mutants of Plexin-B1 significantly increased the rate of migration relative to WT Plexin-B1 ($P \leq 0.05$, Fig. 4a). Mutation of Plexin-B1 also increased cell motility in transwell assays ($P \leq 0.05$) (SI Fig. 10a). Expression of WT Plexin-B1 reduced the invasive capacity of HEK293 cells in invasion assays through matrigel (Fig. 4b). In contrast, expression of four mutant versions of the gene increased the invasive capacity of the cells relative to expression of both WT and vector controls. ($P \leq 0.05$ vs. WT).

Mutation of Plexin-B1 Increases Cell Spreading. Expression of Plexin-B1 also blocks extension of lamellipodia after the plating of fibroblasts on fibronectin, as assessed by the percentage of cell spreading (22). Expression of WT Plexin-B1 in HEK293 cells reduced both the percentage of cell spreading (defined as the percentage of cells $\geq 500 \mu$ m²) and the average cell size 10 min after plating on fibronectin in the presence and absence of Sema4D, relative to a vector control, as expected (Fig. 5a and b and SI Fig. 10b). Conversely, expression of

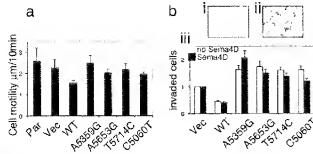


Fig. 4. Mutations in Plexin-B1 increase cell motility and invasion. (a) Rate of cell migration of lentiviral-transduced HEK293 cells expressing WT or mutant Plexin-B1 or transfected with control vector, measured by time-lapse video microscopy. Twenty cells were tracked per experiment, and the numbers represent the average of three independent experiments. The experiment was repeated with an independent lentiviral infection and gave similar results. $P \leq 0.05$, for all mutants vs. WT. (b) (i) and (ii) Photomicrographs of lentiviral-transduced HEK293 cells expressing WT (i) or mutant (A5359G) (ii) Plexin-B1 after invasion through matrigel. (Magnification, $\times 250$) (iii) Relative number of HEK293 cells expressing mutant or WT Plexin-B1 to invade through matrigel-coated invasion chambers after 24 h. Expression of four mutant versions of the gene increased the invasive capacity of the cells relative to expression of WT Plexin-B1 ($P \leq 0.05$). The results are the means of five independent experiments performed in duplicate.

similar levels of mutant Plexin-B1 significantly increased the percentage of cell spreading ($P \leq 0.01$) and average cell size ($P \leq 0.0001$) relative to cells expressing WT Plexin-B1 (Fig. 5a and b and SI Fig. 10b). Addition of *Sema4D* further increased

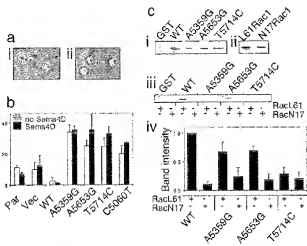


Fig. 5. Mutations in Plexin-B1 increase cell spreading and decrease RacGTP binding. (a) Photomicrographs of lentiviral-transduced HEK293 cells expressing WT (i) or mutant (T5714C) (ii) Plexin-B1 10 min after plating on fibronectin. (Magnification, $\times 400$) (b) Cell spreading. Shown is the percentage of cells $>500 \mu\text{m}^2$ in area, 10 min after plating on fibronectin. $P \leq 0.01$ for all mutants vs. WT, with and without *Sema4D*. Numbers represent the mean of four independent experiments performed in triplicate from two independent lentiviral infections from two different virus preps. A minimum of 320 cells were sized per data point. (c) (i) and (ii) RacGTP binding to Plexin-B1. Shown are the input proteins for GST-pull-down assay. (iii) Coomassie blue staining of GST fusion proteins. (iv) Western blot analysis of cell lysate transfected with the indicated Rac1 proteins and detected by anti-myc. (ii) Lysates of HEK293T cells expressing myc-Rac161 or myc-Rac117 were used in a pull-down assay with GST-Plexin-B1 cytoplasmic domain (mutant or WT). Bound proteins were analyzed by immunoblotting with anti-myc antibody. (b) Band intensities of GST-Plexin-B1 pull-down assays. The results are the average of five experiments \pm SEM normalized to WT with Rac161, $P \leq 0.005$ (A5653G and T5714C) and $P \leq 0.05$ (A5359G) vs. WT.

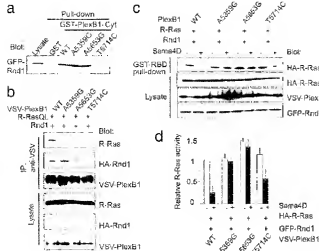


Fig. 6. Mutations in Plexin-B1 inhibit Rnd1, R-Ras binding, and R-RasGAP activity. (a) Interaction between Plexin-B1 mutants and Rnd1. Lysates from COS-7 cells expressing GFP-tagged Rnd1 were used in a pull-down assay with GST or GST-Plexin-B1-Cytm. Bound proteins and total-cell lysates were analyzed by immunoblotting with an antibody against GST. (b) Interaction between Plexin-B1 mutants and R-Ras. Lysates from COS-7 cells expressing the indicated plasmids were immunoprecipitated with an antibody against VSV-G, and bound proteins were analyzed with antibodies against R-Ras, HA, and VSV-G. (c and d). R-Ras GAP activity. COS-7 cells transfected with the indicated plasmids were stimulated with *Sema4D* for 5 min. The cell lysates were incubated with GST-RBD, and bound R-Ras and total-cell lysates were analyzed by immunoblotting. Relative R-Ras activity was determined by the amount of R-Ras bound to GST-RBD normalized to the amount of R-Ras in cell lysates analyzed by NIH Image software. Results are the means \pm SEM of three independent experiments.

lamellipodia extension and average cell size vs. WT ($P \leq 0.01$ and $P \leq 0.0001$, respectively).

Mutation of Plexin-B1 Inhibits Rac Binding. The increase in cell spreading and extension of lamellipodia observed in cells expressing the mutant versions of the protein is a phenotype characteristic of Rac1 activation. WT Plexin-B1 binds to RacGTP (11) and sequesters it from downstream effectors such as PAK (25). Most of the mutations found in prostate cancer fall within the Rac-binding region of Plexin-B1. We therefore tested the binding of three mutant versions of Plexin-B1 to RacGTP. We found that the T5714C mutation inhibited binding of RacGTP to Plexin-B1, and the other two mutations tested, A5359G and A5653G, showed reduced binding, consistent with the cell-spreading data (Fig. 5c).

The T5714C Mutation Inhibits Rnd1 Binding. The small GTPase Rnd1 binds to the cytoplasmic domain of Plexin-B1 and is thought to open up the closed inactive conformation of the cytoplasmic domain of Plexin-B1 (23). To determine whether mutation of Plexin-B1 affects Rnd1 binding, immunoprecipitation assays were performed. The Plexin-B1 mutants A5359G and A5653G were found to bind to Rnd1 to a similar extent as WT. In contrast, the mutant T5714C did not bind Rnd1 (Fig. 6a).

Mutation of Plexin-B1 Inhibits R-Ras Binding and R-Ras GAP-Activating Protein (GAP) Activity. We speculated that the increase in adhesion and loss of cell collapse seen in cells expressing mutant Plexin-B1 may result from a change in signaling through R-Ras. We therefore tested the effect of mutations in Plexin-B1 on R-Ras binding and R-Ras GAP activity.

Using immunoprecipitation assays, we found that the mutants A5359G, A5653G, and T5714C do not bind R-RasGAP (Fig. 6b). Consistent with the lack of R-Ras binding, the A5359G and

AS653G mutants did not show R-RasGAP activity, and R-RasGAP activity was considerably reduced for the T5714C mutant (Fig. 6c and d).

These results show that the three mutants tested have lost or considerably reduced their ability to bind R-Ras and inactivate it. Cells expressing mutant Plexin-B1 will therefore have an increased ratio of active/inactive R-Ras relative to cells expressing WT Plexin-B1. Activated R-Ras activates integrins resulting in an increase in cell adhesion and motility. The finding of a loss of R-RasGAP activity for mutant Plexin-B1 is therefore consistent with our *in vitro* assays.

Discussion

We have found a high frequency of somatic missense mutations in the Plexin-B1 gene in localized and metastatic prostate cancer. The high proportion of nonsynonymous sequence changes found implies that these changes contribute to tumor progression and are not incidental passenger mutations. Furthermore, the mutations tested altered the function of the Plexin-B1 protein, increasing cell motility, invasion, adhesion, and lamellipodia extension and decreasing collapse relative to WT Plexin-B1. The mutations inhibit or hinder Rac1 and R-RasGTP binding and R-RasGAP activity. Overexpression of Plexin-B1 protein in primary prostate cancers was also seen. Together these results suggest that Plexin-B1 has a role in prostate cancer progression.

A standard method of identifying oncogenes is to transfect the gene construct into NIH 3T3 cells and measure tumorigenicity *in vivo*. However, such *in vivo* studies have not been feasible because of an inability to maintain exogenous expression of Plexin-B1 over time and will require the use of inducible vectors.

Plexin-B1 activates the oncogenes c-Met and ErbB2 (13, 14), resulting in an increase in cell motility and promotion of the invasive phenotype (14). High levels of c-Met and ErbB2 protein have been observed in prostate cancer (26, 27), and we have now found that Plexin-B1 and Sema4D are also overexpressed. Overexpression of WT Plexin-B1 in prostate tumor cells may promote the invasive phenotype through activation of c-Met and ErbB2. The mutations are less likely to affect the activating pathways mediated by Plexin-B1 via c-Met and ErbB2, because these proteins interact with the extracellular domain of Plexin-B1, whereas the mutations are in the cytoplasmic domain. The mutations may therefore selectively inhibit Plexin-B1 inhibitory pathways (via K-Ras and Rac) while retaining activation of c-Met and ErbB2, resulting in an increase in adhesion, motility, and invasion. In addition, a significant increase in cell spread was observed in cells expressing mutant Plexin-B1 relative to vector controls. This finding indicates that the mutations may confer a gain of function to the cell in addition to inactivating inhibitory pathways.

Some of the mutations were present in many tumors. The high frequency of mutations at specific sites suggests specific roles for the mutated regions or specific residues in the neoplastic process. Mutational hotspots occur in several other oncogenes such as TP53, RAS, and BRAF (28–30). Some of the tumor samples had up to three mutations in the Plexin-B1 gene. Different mutations may occur in distinct groups of cells or foci. Prostate cancer is genetically heterogeneous with many overlapping foci, each with a distinct genotype, in one tumor. Up to three different mutations were found in the KLF6 gene in single foci of prostate cancer (18), and up to four mutations were found in the androgen receptor gene (19). If present in the same cell, the mutations may destabilize the inactive closed conformation formed between the C1 and C2 regions of the cytoplasmic domain of Plexin-B1 (23). Increasing numbers of mutations would have a cumulative effect on the protein, further destabilizing the closed conformation and offering a greater selective advantage to the cell. The frequency of mutations in Plexin-B1 in primary tumors exceeds the clinical risk of prostate cancer metastasis, suggesting that other factors are important in addition to promote metastasis.

Because prostate tumors often contain overlapping foci of different genotypes and many nonneoplastic cells, the method used to isolate tumor cells for mutation detection is important. Prostate cancer tissue isolated by laser capture microdissection (LCM) provided DNA of sufficient purity for mutation detection by direct sequencing. In contrast, tissue isolated by manual microdissection provided DNA of lesser purity, and mutations were only detected by SSCP analysis and restriction-enzyme digestion. It is likely that mutation detection in DNA from nonmicrodissected prostate cancer tissue will prove more problematic.

Our data indicate that Plexin-B1 is one of the most frequently mutated genes thus far found in prostate cancer. Plexin-B1 is likely to be a key player in cancer invasion and metastasis and is a potential target for anticancer therapy.

Methods

Tumor Tissue. Tissue was obtained by LCM for the metastases and for fresh frozen prostate tissue and by manual microdissection for the paraffin-embedded tissue from primary cancers. Areas of cancer were identified by a consultant histopathologist (A. Freeman, University College London). Samples were collected with local ethical board approval.

Immunohistochemistry. Sections from four replicate TMA blocks of 85 primary prostate cancers and 85 samples of nonneoplastic prostate tissue from the same radical prostatectomies were stained with antibodies to Plexin-B1 (H300; SantaCruz), Sema4D (610671; BD Biosciences), c-Met (c-28; Santa Cruz), or with hematoxylin and eosin. The sections were scored blind by a consultant histopathologist according to the extent and intensity of staining. Positive staining was defined as unequivocal staining of at least 75% of epithelial or cancer cells in at least one of the four cores.

Screening for Mutations. SSCP analysis was carried out as described in ref. 31; see *SI Methods* for details. Numbering for the mutations is according to GenBank accession no. AB007867. Control DNA from unrelated individuals of Caucasian origin was screened for the presence of each identified mutation by PCR and restriction enzyme digestion (31). See *SI Methods* for details.

RT-PCR. Real time RT-PCR was performed on an ABI PRISM 7700 (Applied Biosystems) by using β -actin as control and primers and probe spanning exons 31 and 32 of the Plexin-B1 gene.

In Vitro Mutagenesis. Plexin-B1 in pcDNA3 was kindly provided by A. Hall (A. Hall, Memorial Sloan-Kettering Cancer Center, New York, NY). The AS359G, AS653G, TS714C, and CS060T sequence changes were introduced using the QuikChange kit (Stratagene). See *SI Methods* for primer sequences. The sequence was checked by DNA sequencing, and protein expression was confirmed by Western blot analysis and immunocytochemistry.

Lentiviral Transduction. WT Plexin-B1 and the four mutant constructs were cloned into pLenti/V5-D-TOPO, and the sequence was verified. Virus, produced in 293FT cells, was transduced into HEK293 cells with polybrene (8 μ g/ml). Infected cells were selected with blasticidin. Protein expression was confirmed by Western blotting and immunocytochemistry.

Cell Culture. LNCaP, PC3, and DU145 cells were grown in RPMI medium 1640 with 10% FCS. HEK293 and COS-7 cells were grown in DMEM with 10% FCS.

Preparation of Recombinant Sema4D-Fc. The Sema 4D-Fc construct was kindly provided by Hitoshi Kikutani (Osaka University, Osaka, Japan) in vector pEFBos. Recombinant Sema4D-Fc was purified from conditioned medium of COS-7 cells transfected with pEFBos (Sema4D-Fc) by using protein A Sepharose CL4B beads and was

concentrated using Centricon columns (Amicon). The protein concentration and purity were assessed by Western blotting compared with known concentrations of Sema3A-Fc (R & D Systems).

Cell-Substrate-Adhesion and Cell-Spreading Assays. Cells were plated onto the lids of 96-well plates (1×10^4 cells per well) coated with 20 μ g/ml fibronectin or poly-L-lysine with or without Sema4D (100 ng/ml), incubated at 37°C for 10 or 30 min, washed with PBS, fixed, and stained with 0.5% crystal violet. Staining was measured on a plate reader at 595 nm. Results shown are the mean of four independent experiments performed in triplicate with two different lentiviral transductions \pm SEM.

For the cell-spread assays, cells were plated on 20 μ g/ml fibronectin with or without Sema4D (100 ng/ml), incubated at 37°C for 10 min, washed, fixed, and stained with 0.5% crystal violet. Photomicrographs of cells were sized using Andor imaging systems. A minimum of 320 cells were sized per data point. Results shown are the mean of four independent experiments performed in triplicate with two different lentiviral transductions \pm SEM.

Cell-Collapse Assay. COS-7 cells were transiently transfected with Plexin-B1 and Rnd1-myc and stimulated with Sema4D (100 ng/ml) for 5 min, 16 h after transfection. Cells were fixed in 4% paraformaldehyde and stained by immunofluorescence with Plexin-B1 (H300; Santa Cruz) and anti-myc (9E10; Santa Cruz) antibodies and phalloidin-TRITC (Sigma-Aldrich). The degree of collapse (percentage of cells $<500 \mu\text{m}^2$) was determined by sizing transfected cells using Andor imaging. Fifty cells were counted per experiment. Results are the mean of three independent experiments \pm SEM.

Cell-Motility Assays. Time-lapse video microscopy was performed 24 h after plating the cells in a humidified, 5% CO₂ atmosphere at 37°C. Images were captured at 10-min intervals over 16 h for each cell type. The experiment was independently repeated three times, and 20 cells were tracked in each experiment by using Motion Analysis (Kinetic Imaging). The average rate of cell migration was determined with Mathematica software (Wolfram Research).

Invasion Assays. Serum-starved lentiviral-transduced HEK293 cells (6×10^4 cells per insert) in DMEM were placed in the upper chamber of a 24-well, 0.8- μm BD Biocoat matrigel invasion chambers (BD Biosciences) with or without purified Sema4D (100 ng/ml). DMEM with 10% serum was placed in the lower chamber. The chambers were incubated at 37°C for 24 h. Cells on the upper side were removed, and those on the underside were fixed, stained with crystal violet, and counted by using a microscope. Five independent experiments were performed in duplicate.

GST-B1cyto Purification. The region of Plexin-B1 cDNA encoding the intracellular domain (amino acids 1,512–2,135; GenPept accession no. NP_002664) was amplified by PCR (see primer sequences in *SI Methods*). The PCR product was cloned into pGEX-IT-3 (Amersham) by SalI and XhoI sites to produce pGEXB1cytoWT. Mutations were introduced into pGEXB1cytoWT using QuikChange II XL *in vitro* mutagenesis kit (Stratagene). GST-B1cyto fusion proteins were produced in *E. Coli* BL21 and purified with glutathione Sepharose 4B (Amersham). The bound proteins were analyzed by Coomassie blue staining in SDS/8% PAGE gel.

Rac-Binding Assays. GST-Plexin-B1-coated Sepharose beads were incubated with HEK293T cell lysate expressing constitutively active Rac1.61 or dominant negative RacN17. The bound protein was analyzed by Western blot analysis.

Rnd1-Binding Assays. GST-Plexin-B1-coated Sepharose beads were incubated with COS-7 cell lysate expressing Rnd1. The bound protein was analyzed by Western blot analysis.

R-Ras-Binding Assays. COS-7 cells transiently transfected with VSV-Plexin-B1, HA-Rnd1 and R-RasQL were lysed in cell lysis buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM MgCl₂, 1% Nonidet P-40, 10% glycerol, 1 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 mM DTT]. The lysate was incubated with 4 μ g/ml of monoclonal antibody against VSV (PSD4; Sigma) for 2 h and subsequently incubated with protein G-Sepharose beads (GE Healthcare) for 1 h. The bound protein was analyzed by Western blot analysis.

R-RasGAP Activity. Transiently transfected COS-7 cells were stimulated with Sema4D for 5 min, and lysed in cell lysis buffer [25 mM Hepes-NaOH (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.25% Na-deoxycholate, 10% glycerol, 10 mM MgCl₂, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin]. Lysates were incubated with 75 μ g of GST-fused Ras-binding domain of c-Rac-1 and analyzed by immunoblotting.

Statistical Analysis. For cell-function assays, *P* values were calculated using a two-tailed Student's *t* test, except for Rac binding assays, for which a one-tailed test was used. The Mann-Whitney test (two-tailed) was used for the immunohistochemistry results.

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